IB/2004/04073

PA 1261050

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

December 16, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/528,596* ✓
FILING DATE: *December 10, 2003* ✓

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

Trudie Wallace
TRUDIE WALLACE
Certifying Officer

324 U.S.PTO

By Express Mail # EL997184508US

121003

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket #: 5463-2P	Type a plus sign (+) inside this box→	+

INVENTOR(S)/APPLICANT(S)	
NAME *(First, Middle, Last)*	RESIDENCE *(City and either State or Country)*
Shen SHUO	Singapore
Hong WANJIN	Singapore
Lim-Seng GEE	Singapore
Tan-Yee JOO	Singapore
Burtram Clinton FIELDING	Singapore
Goh Phuay YEE	Singapore
Timothy Tan Hoe PENG	Singapore
Fu JIANLIN	

TITLE OF THE INVENTION *(280 characters max)*

MATURATION OF THE SPIKE (S) PROTEIN OF SARS CORONAVIRUS AND USES THEREOF

CORRESPONDENCE ADDRESS

Kent H. Cheng, Esq.
(212) 687-2770

Cohen, Pontani, Lieberman & Pavane
551 Fifth Avenue, Suite 1210
New York, New York 10176

ENCLOSED APPLICATION PARTS *(check all that apply)*

[x]	Specification	*Number of Pages* [17]	[]	Other *(specify):*
[x]	Drawing(s)	*Number of Sheets* [14]		

METHOD OF PAYMENT *(check one)*

[x]	A check is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNTS: **$80**
[x]	**If no check is enclosed or the enclosed check is insufficient** - The Commissioner is hereby authorized to charge the filing fees or credit any overpayment to Deposit Acct. No. 03-2412.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government

[x] No

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _

[x] Small Entity Status is claimed

Respectfully submitted,

Dated: December 10, 2003

COHEN, PONTANI, LIEBERMAN & PAVANE
551 Fifth Avenue, Suite 1210
New York, New York 10176
(212) 687-2770

By: Kent H Cheng

Kent H. Cheng
Reg. No. 33,849

PROVISIONAL APPLICATION FILING ONLY

Rev. Jun-02

MATURATION OF THE SPIKE (S) PROTEIN OF SARS CORONAVIRUS AND USES THEREOF.

FIELD OF THE INVENTION

The invention relates to the use of a matured, glycosylated Spike (S) protein of SARS Coronavirus and a method for producing the same.

BACKGROUND OF THE INVENTION

In 2002-2003, a new virus that caused an atypical pneumonia, a disease termed SARS, emerged from the Southern part of China and infected people from at least 30 countries. At the end of the outbreak in July 2003, the consolidated World Health Organization number of reported patients and deaths due to SARS, was about 8500 and more than 800 respectively. This outbreak not only incurred great social and medical costs to affected countries, it also had devastating effects on regional as well as global economies.

A novel coronavirus was established to be the causative agent for SARS (Drosten et al., 2003; Ksiazek et al., 2003) and was subsequently named SARS coronavirus or SARS CoV. It's genome of 29.6kb revealed 14 open reading frames (orfs), encoding the replicase, spike, membrane, envelop and nucleocapsid (N) which are similar to other coronaviruses, and several other unique proteins (Marra et al., 2003; Rota et al., 2003).

The coronavirus spike (S) proteins are very large viral surface proteins (~150 kDa) and belong to type I transmembrane glycoproteins. In the coronavirus family, the S proteins define the viral tropism by their receptor specificity and their membrane fusion activity during virus entry into cells (Gallagher TM & Buchmeier MJ, 2001). Therefore, the spike proteins represent potential targets for vaccine and drug design to inhibit the coronavirus entry into host cells. The SARS CoV spike protein has been isolated (Spiga et al., 2003). The difficulty with the SARS CoV spike protein is that being a glycol protein it is difficult to produce enough of the protein in a humanized form that would be suitable for the production of antibodies, vaccines and other therapeutic, diagnostic and prophylactic tools. A protein in a humanized form is one that is similar to the protein form in a human body.

A solution to this difficulty would be to find a system capable of producing a SARS CoV spike protein that is glycosylated so as to be humanized.

SUMMARY OF THE INVENTION

According to the first aspect of the present invention there is provided a mature, glycosylated spike protein of a coronavirus.

A second aspect of the invention provides a method of producing a mature, glycosylated spike protein of a coronavirus comprising the steps;

a) transfecting a cell with a nucleic acid encoding a spike protein of a coronavirus or part there of,
b) expressing the spike protein in the cell, and
c) isolating the spike protein.

A third aspect of the invention provides a method of screening for a mature, glycosylated spike protein of a coronavirus comprising the steps;

a) Isolating a spike protein
a) Immunoprecipitating the isolated spike proteins with Endo-H
b) Detecting the remaining spike proteins that are the mature glycosylated spike protein.

A fourth aspect of the invention provides an antibody to a mature, glycosylated spike protein of a coronavirus or part their of.

The following statements relate to the first to fourth aspects of the invention.

In one embodiment, preferably the coronavirus is a SARS coronavirus. Preferably the coronavirus is a SARS coronavirus strain, 2774.

In another embodiment, preferably the mature glycosylated spike protein contains a transmembrane domain (TMD).

In another embodiment, preferably the mature glycosylated spike protein is a 210KDa protein.

In another embodiment, preferably the cell is a lung cell line A549.

In another embodiment, preferably the antibody is used for immunodectection of a SARS coronaviral infection.

In another embodiment, preferably the spike protein or the antibody is used in the production of a vaccine.

A BRIEF DISCRIPTION OF THE FIGURES

Figure 1 Diagram of the full-length, C-terminally-truncated and internally-deleted proteins expressed in E.coli and mammalian cells. Viral RNA was extracted from vero E6-adapted strain 2774 and RT-PCR was performed using primers listed in Table 1. The amino acid positions of individual proteins are indicated. (a) S.1, S.2, S.3, S.9 and S.10 were expressed in BL21(DE3) as the N-terminal GST fusion proteins using pGEX41 based plasmids as described in Methods. (b) S-HA, S.4, S.5, S.6, S.7 and S.8 were expressed in mammalian cells as the C-

terminal HA-tagged proteins using pXJ3'-HA based plasmids, which could be detected by both anti-HA and anti-S antibodies. (c) S-c15, S.11, S.12, S.13, S.14, S.15 and S.16 were expressed in mammalian cells as untagged proteins using pKT0 based plasmids as described in Methods. (d) S.17, S.18, S.19 and S.20 are internal-deletion mutants without amino acids 601 to 800, 401 to 600, 201 to 400 and 31 to 200, which are pXJ3'-HA-based plasmids. SP, signal peptide; CCR, coiled coil region; HR1 and 2, heptat repeat 1 and 2; TMD, transmembrane domain; CD, cytoplasmic domain.

Figure. 2 The S specific antisera recognizing the full-length and truncated S proteins. Antisera Rb-á-S.1, Rb-á-S.2, Rb-á-S.3, Rb-á-S.10 and Horse-á-S (panels A, B, C, D and F) were used, as indicated on the top, in immunoprecipitation to test the full-length and truncated S proteins expressed and radio-labeled in Cos-7 cells. Numbers on the left indicate molecular masses in kilo Daltons. An antiserum P6 from recovered patient 6 was used as a positive control (panel E). The S protein of IBV was also expressed and could be recognized by rabbit á-IBV antibodies but not the antibodies against the S of Sars (data not shown). coronavirus. Lysate of Cos-7 cells, infected with V/T7 virus and mock-transfected, were used as a negative control.

Figure. 3 Maturation of S in transfected Cos-7 cells. The full-length S construct was transfected in V/T7 infected cells. The cells were radio-labeled 3h post-transfection for 1h and chased for 6h. At each time point, one of 60mm dishes was removed from 37$_0$C incubator. (a) Rb-á-S.3 (panel A) and Rb-á-S.10 (panel B) were used for immunoprecipitation of the S protein. Samples were separated in 7.5% SDS-PAGE gels. (b) The same samples were immunoprecipitated with Rb-á-S.3, washed with RIPA buffer 3 times. The proteins were resuspended in 40 µl of digestion buffer, and equal volume of which was treated or mock-treated with Endo-H for 3h at room temperature. The samples were loaded on a 7.5% SDS-PAGE gel.

Figure. 4. Endo-H treatment and surface expression of the full-length and internally-deletion and C-terminally-truncated S proteins. (a) The S constructs as indicated on the top were transfected into Cos-7 cells. The cells were labeled with ^{35}S-Met and -Cys for 3h and 4 times of cold Met and Cys were added to medium to chase overnight. Samples were immunoprecipitated, endo-H-treated as described in the legend of Fig. 3. The treated and more-treated samples were separated on a 7.5% SDS-PAGE gel. (b) The plasmids with (pKT-S-c15) and without transmembrane domain (pKT-S.12) were transfected into Vero E6 (panels A and B) and Cos-7 cells. The cells were incubated, 20h post-transfection, with Rb-á-S.3 and Rb-á-S.10 (1:40 each) for 1.5h, followed by 1.5h incubation with goat á-rabbit, FITC-conjugated IgG.

Figure. 5. Co-immuno precipitation of S with host 62- and 64-kD proteins. (a) The full length (S), C-terminally-truncated (S.4 to S.8) and internally deleted S proteins (S.17 and S.20) were expressed and labeled with ^{35}S-Met and Cys in

Cos-7 cells as indicated on the top of gel. Cell lysates were immunoprecipitated with Rb-á-S.1 (left panel) and Rb-á-S.2 (right panel). (b) Cos-7 cells were transfected or co-transfected with pKT-0 based plasmids containing the S, M, and E genes as indicated. The radio-labeled cell lysates were immunoprecipitated with rabbit anti-S, anti-M anti-E or combination of the 3 antibodies as indicated. The proteins were separated on a 5-15% gradient SDS-PAGE gel. Molecular masses of markers (M, in Kilo Daltons) were indicated on the left. The S, M, E and host proteins are indicated on the right. (c) The full-length (S) and C-terminally truncated S (S.13 and S.15) were expressed in Vero E6, 293T (left panel), A549 and MRC-5 (right panel) cells. The radio-labeled proteins were immunoprecipitated with Rb- á-S.1 and separated on a 7.5% SDS-PAGE gel. Two sets of molecular markers were used containing either 220kD or 200kD marker as indicated on the left. Two forms of the full-length S proteins and host proteins are indicated on the right.

Figure. 6 Cleavage of S in transfected Cos-7 cells. (a) The full-length (S) and C-terminally truncated S proteins (S.11 to S.16) were expressed in Cos-7 cells and western blot were performed using Rb-á-S.1, Rb-á-S.2, Rb-á-S.3 and Rb-á-S.10 as indicated. (b) The full-length (S) and C-terminally truncated S proteins (S.13 and S.15) were expressed in Vero E6, 293T, A549 and MRC-5 cells and western blot were performed using Rb-á-S.1 and Rb-á-S.2 as indicated. For negative control, the cells were infected with V-T7 but mock-transfected. For loading control, the same membranes were striped and re-probed with mouse-á-actin.

Figure. 7 Cleavage of S in virus-infected cells. Vero E6 cells were infected with Sars coronavirus 2774. Cells and supernatant were harvested in 1 and 5 x RIPA buffer, respectively, 20h post-infection. (a) Western blot was performed using Rb-á-S.3, Rb-á- S.10 as indicated. (b) Immunoprecipitation was performed using Horse-á-S (left panel) and patient serum P6 (right panel). The immunoprecipitated proteins were then used for western blot, using the mixture of Rb-á-S.3 and Rb-á-S.10. Specific bands of S are indicated on the left and inside gels by arrows. For accuracy of size calculation, 3 sets of pre-stained protein markers were used and their sizes in Kilo- daltons are indicated on both sides.

A DETAILED DISCRIPTION OF THE INVENTION

MATERIALS AND METHODS

Cells and viruses

Vero E6 and Cos-7 cells (American Type Culture Collection) were maintained in DMEM medium (GIBCO, BRL), supplemented with fetal calf serum (10%), streptomycin (1,000 ug/ml) and penicillin (1,000 units/ml). The Singapore stain 2774 of Sars coronavirus was isolated in Tan Tock Seng Hospital and adapted to grow in Vero E6 cells in laboratory of Environmental Health Institute (EHI),

Singapore. Passage 3 in Vero E6 cells were used for direct RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR) and sequence analysis. Recombinant vaccinia/T7 virus (VT3) was grown and titrated on Vero cells, which is a subclone for growth of avian infectious bronchitis coronavirus, IBV (Shen and Liu, 2003).

RT-PCR and sequencing

Viral RNA was extracted from strain 2774-infected cells, using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions in laboratory at EHI. RT-PCR was performed using the Expand Reverse Transcription and High Fidelity PCR Kits (Roche). Annealing and extension times were optimized for amplification of PCR products with different sizes using different primers. Specific primers were used for amplification, sequencing and cloning. Automated sequencing was carried out using PCR products or cDNA clones and specific primers as previously described (Shen *et al.*,2000). Sequence analysis was carried out using the GCG suite of programs.

Construction of plasmids

Forward primer SS03-56 and backward primers SS03-64 and SS03-57 were designed to amplify the S gene with and without HA-tag. The PCR products were digested with BamHI and StuI and ligated into BamHI/EcoRV-cut pXJ3'-HA (ref) and BglII/EcoRV-cut pKT0 (Liu *et al.,* 1994), resulting in plasmids pXJ-S, pXJ-S-HA and pKT-S. Primers used in this study are listed in Table 1. Forward primer SS03-56 and backward primers SS03-70, SS03-71, SS03-89, SS03-90 and SS03-91 were designed to obtain five PCR products covering Sars coronavirus 2774 sequence from nucleotides 21476-22864, 21476-23992, 21476-25060, 21476-24415 and 21476-23866, respectively. Each of the 5 PCR products was digested with BamHI and EcoRV or StuI, and then ligated into pXJ3'-HA digested with the same enzymes, giving rise to pXJ-S.4-HA, pXJ-S.5-HA, pXJ-S.6-HA, pXJ-S.7-HA and pXJ-S.8-HA. Encoding regions of these constructs are indicated in Fig.1a. These 5 fragments of the S gene are placed under the control of a T7 promoter and a CMV EI promoter.

Forward primer SS03-56 and backward primers SS03-111, SS03-112, SS03-113, SS03-114, SS03-115 and SS03-116 were designed to amplify 2774 specific sequence from nucleotides 21476 to 25171, 25066, 24934, 24415, 24157 and 23866, respectively. They were digested with Bam HI and ligated to BglII/BamHI-cut pKT0 and placed under the control of a T7 promoter, giving rise to plasmids pKT-S.11, pKT-S.12, pKT-S.13, pKT-S.14, pKT-S.15 and pKT-S.16. Encoding regions of these constructs are indicated in Fig.1b.

Plasmids pGST-S.1, pGST-S.2, pGST-S.3, pGST-S.9 and pGST-S.10 were constructed by cloning BamHI/XhoI-digested PCR fragments into BamHI/XhoI-cut pGEX4T1 (ref ?). The five fragments of the S gene were fused at the 5'-end with a sequence encoding GST protein. The 3'-end sequences of each fused gene encode part of the S protein as indicated in Fig 1c.

Purification of the recombinant S proteins expressed in *E.coli.*

Plasmids pGST-S.1, pGST-S.2, pGST-S.3, pGST-S.9 and pGST-S.10 were transformed into BL21 (DE3) cells, respectively. A single colony from each plate was grown at 37oC overnight in LB agar-plate containing ampicillin (100 µg/ml). Five milliliters of the resulting cultures was inoculated into 2 liters of LB medium containing ampicillin (100 µg/ml) and was incubated in a shaker at 37oC until OD_{600} reached to 0.6. Expression of proteins was induced using 1mM IPTG. Cells were harvested 2 h after induction by centrifugation at 5,000 g for 10 min at 4oC. The cell pellet was resuspended in PBS-1mM PMSF-20µg/ml Dnase I and lysed by two passages through a French Press. Lysate was centrifuged at 22,000 g for 30 min. The insoluble proteins in pellet was washed 3 times and resuspended in PBS containing 1% triton X-100. Proteins were separated in 10% PAGE-SDS gels. Gel strip containing GST-fusion protein was cut and the proteins were eluted using Mini Trans-Blot cell (BioRad). The resulting fusion proteins were detected in Western Blot using mouse anti-GST antibodies and their concentrations were estimated by comparison with BSA standards in Commassie bluestained PAGE gel.

Expression of the S protein in mammalian cells

Fifty percent of confluent monolayer of cells in 60 mm Petri dish was infected at multiplicity of infection (MOI) of 1 with recombinant vaccinia/T7 viruses (vTF7-3) that express bacteriophage T7 RNA polymerase. After 1-hour adsorption, cells were transfected with 2 to 5µl of plasmid using Effectene Reagents (Qiagen) according to manufacturer's instruction. Transfected cells were incubated overnight at 37oC and cell lysate was prepared by resuspensing cell pellet in 1 x protein loading buffer for Western Blot. For melabolic labeling, 3 to 4 h after transfection cells were starved with methionine- and cysteine-free medium (DMEM, NEN) for 0.5 h and then labeled for 1 to 2 h by replacing with the same medium supplemented with ^{35}S-Met and ^{35}S-Cys (100 Ci/ml, NEN). Cells were either lysed immediately or chased with 4 mM methionine and cysteine for various time as indicated before lysis with ice-cold lysis buffer.

Generation of antisera against the S protein

To raise antibodies against the S protein, the five recombinant GST-fusion proteins described above were used to inject rabbits and mice as describe previously (ref). The final bleeding (after 7 to 12 boosting) was carried out 10 days after last immunization. Antisera were adsorbed with individual or mixtures of Vero E6, Cos-7 and 293T cells. Specific IgGs were also purified from the antisera collected. The specificity and titers of rabbit and mouse antisera were tested by immunoprecitation (Fig. 2), Western blot, and IF (data not shown) with the S protein from infected and/or transfected cells.

Endoglycosidase (Endo-H) treatment of the S proteins

Cells were transfected or mock-transfected with plasmids as described above. After 4 h incubation, cells were metabolically radio-labeled with [^{35}S]-

methionine/[35S]-cysteine as described previously (25). A portion of cell lysates was immunoprecipitated with anti-S serum and protein A-agarose beads (Roche). The pellets were washed three times with standard RIPA buffer, dissolved in 20 µl of digestion buffer (50 mM Tris, pH 6.8, 0.25% SDS) and incubated at 95oC for 5 minutes. Ten µl of the supernatant were mixed with 10 µl of digestion buffer with or without Endo-H (0.2 mU, Boehringer) and incubated for 4 h at 37oC.

Transcription and translation *in vitro*

Transcription and translation *in vitro* were performed using T7 RNA polymerase and wheat germ lysates (Promega) as previously described (Shen *et al.*, 1999). *In vitro* transcription and translation were carried out separately to assess the quantity of transcripts and efficiency of translation of each gene in each construct. The synthesized proteins were radio-labeled with S35-methionine. Aliquots of the products were analyzed by 17.5% SDS-PAGE and detected by autoradiography as previously described (Shen, *et al.*, 1999).

Immunofluorescence and confocal microscopy

Cells grown on chamber slide (Iwaki) were infected with recombinant vaccinia/T7 viruses. After incubation at 37oC for one hour, cells were transfected with appropriate plasmid DNA using Effectene Transfection Reagent (Qiagen). At different time points, cells were fixed in 4% Para formaldehyde-PBS for 15 minutes and permeabilized with 0.2% Triton X-100-PBS for 10 minutes. Immunofluorescence staining was performed by incubating cells with anti-serum (diluted 50 to 100 fold) for 1h and subsequently with FITC- or Rhodamin-conjugated goat anti-mouse/anti-horse or goat anti-rabbit antibodies (1:500 to 1:1000, Dako) for 1 h. Both primary and secondary antibodies were diluted in PBS containing 1% BSA. Cells were rinsed three times with PBS after each step and the cells in the chamber slides were observed with a Zeiss confocal microscope and scanned with a connected Bio-Rad MRC1024 scanner.

RESULTS

Specificity of antibodies against the S protein raised in animals

Five (á-S.9 is not ready) GST fusion proteins covering different region of S (Fig. 1a) were used to immunize white New Zealand rabbits. Specificity and sensitivity of antisera against S were tested after 3, 5, 7 and 11 boosters by means of immuno precipitation, western blot and IF experiments using cell lysates harvested 16 h after transfection of the S constructs (Fig. 1c) in Vero E6 and Cos-7 cells. As shown in Fig.2 in an IP experiment, as expected, rabbit á-S.1, á-S.2 and á-S.3 specifically bind the full-length (S) or C terminally truncated S proteins (S.11 to S.16). Rabbit á-S.10 only recognize the full length and largest S proteins (S and S.11 to S.13) but not the smaller one (S.14 to S.16). The size of each protein is the same as those detected using serum from a recovered patient (P6) and antiserum raised in horse, immunized with E. coli-expressed fusion protein overlapping with S.2 (a region from amino acids 460 to 820). When the

full-length S protein was used in IP experiments (overnight radio-labeled), a 210 kD smear band was observed just above a 200kD band. We also noticed that two proteins with a size of 62kD to 64kD were co-immuno precipitated with the S proteins. We believe these two proteins are specifically interacting with S, because no similar bands were immuno precipitated in mock-transfected cells and cells expressing the IBV S protein. These specific interactions will be further described and discussed in the subsequent section.

Maturation of the Sars coronavirus S protein in transfected cells

To characterize the two forms of the S protein described above, pulse-chase experiments were performed in a vaccinia-T7 expression system (ref). One hour post-infection with V/T7, Cos-7 cells were transfected with the plasmid pKT-Sc15 containing the full-length S gene. Three hours post-transfection, cells were starved for half hour and radio-labeled with [^{35}S]-methionine and cysteine for 1 h. Cold methionine and cysteine were added to 4 mM and samples were collected at different time points as indicated in Fig. 3. Immunoprecipitation was performed using antisera Rbá-S.3 and Rbá-S.10, against the N- and C-terminal region of S. As shown in Fig. 3a, no 210kD form of S was observed at time zero (1 hour labeling before chase), but the 210kD band appeared after 30 min and became more intense till the end of pulse-chase experiment, indicating an increasing amount of 210kD proteins were formed. In contrast, the amount of 200 kD protein decreased as pulse-chase experiment proceeded. The results suggested that 200 kD S protein is core-glycosylated form, while the 210 kD protein is the matured, fully glycosylated form of S. To support this conclusion, the time course samples were immunoprecipitated and treated with Endo-H, which would remove immature glycons of N-linked glycoproteins. Indeed, as shown in Fig. 3b, the 210 kD protein was resistant to Endo-H digestion, while the 200 kD protein was Endo-H sensitive. The results confirmed that the full-length, matured S protein of Sars coronavirus is 210 kD in transfected cells.

Transmembrane domain is essential for maturation of S

Next, the maturation of S was further investigated using the full-length and truncated S constructs. The S gene was truncated from the C-terminus systematically (Fig. 1c), giving rise to S.11 to S.16, without cytoplasmic domain (-CD, S.11), transmembrane domain (TMD, -TMD/-CD, S.12), heptad repeat 2 (HR2, S.13, -HR2/-TMD/-CD), heptad repeat 1 (HR1, S.14, -HR1/2/-TMD/-CD), coiled-coil region (CCR, S.15, -CCR/- HR1/2/-TMD/-CD) and C-terminal 458 amino acid deletion (-C458, S.16, -CCR/-HR1/2 /-TMD/-CD). The plasmids were transfected into V/T7-infected Cos-7 cells. Cell lysates were immunoprecipitated with horse anti-S serum, divided into two aliquots and treated or mock-treated with Endo-H. As seen in Fig.4, only the full-length S and the truncated S.11 without CD were Endo-H resistant. In contrast, other C-terminally truncated S proteins (S.12 to S.16), all of them without the TMD, were Endo-H sensitive. Furthermore, two (or four) of other truncated S proteins, S.17 and S.20 (or S.17 to 20) with deletions from amino acid 31 to 200 and 601 to 800, respectively, were Endo-H resistant (Fig. 5). In all cases, the Endo-H resistant forms of S are

larger and migrate lower than the Endo-H sensitive forms. The results indicated that the TMD is an essential domain for the complete glycosylation and maturation of S and that only those containing TMD could be transported to the trans-Golgi apparatus and processed to the matured form of S.

Transmembrane domain is essential for cell surface expression of S

The full length S protein, C-terminally-truncated and deletion mutants of S were expressed in Cos-7 and Vero E6 cells. Surface expression of these proteins was tested with specific anti-S antibodies in IF experiments. The results showed that only those S proteins with TMD were expressed on the cell surface 24 hours after transfection. A representative IF experiment was shown in Fig. 4b. The full-length S and S.17 proteins were expressed on the cell surface, while S.6 without TMD was not expressed on the surface 24 h after transfection. The results are in consistence with those of the S maturation, indicating that only S proteins with TMD could be transported to the trans-Golgi, fully glycosylated and expressed on the cell surface.

Two host proteins, 62kD and 64kD, interact and co-immuno precipitate with the S protein

As previously shown in Fig. 2, 3a, 3b and 4a, two host proteins were co-immuno precipitated with the full-length, C-terminally truncated and deletion mutant S proteins when rabbit, horse against S and patient serum were used. These two proteins were sensitive to Endo-H treatment (Fig. 3b and 4a). They bind specifically to the S protein of Sars coronavirus, but not that of IBV (Fig. 2, panel A, B and E, and Fig. 4a). More importantly, we proved that they bind to the N-terminal region of S as the deletion mutant S.20 lacking the amino acid residues from 31 to 200 did not bind to them (Fig. 5a). In contrast, other S proteins, including the shortest one S.4 containing only the first 463 amino acids, bind to them. Furthermore, in co-transfection of S with M and E constructs (Fig. 5b), it was clearly shown that only the anti-S antibodies can co-immuno precipitated these two proteins with S but not the anti-M and anti-E antibodies. When cells were co-transfected with S, M and E, the anti-S antibodies still co-immuno precipitated these two host proteins. In another word, the E and M did not compete with S for specific binding or inhibited S from binding to them.

In order to investigate whether similar host proteins in other monkey kidney and human kidney and lung cells interact with S, Vaccinia/T7 expressions were performed in Vero E6, 293T, A549 and MRC-5 cells using the full-length S and truncated S.13 and S.15 constructs. Vero E6 is a monkey kidney cell line in which 2774 has been adapted to grow. 293T is human kidney cell line and other two are human lung cell lines. The cell lysates were immunoprecipitated with specific anti-S antibodies (Fig. 5c). The results showed that the 62kD and 64kD proteins existed not only in monkey kidney cell Vero E6, but also in human cells 293T, A549 and MRC-5. They bond specifically to the S protein of Sars coronavirus. Interestingly, when expressed in A549 cells the major band is the matured 206kD form, but in other cells the 195kD immature protein dominates.

Cleavage of the S protein in transfected and infected cells

Finally, using our anti-S antibodies, we were able to determine if the S protein of Sars coronavirus was cleaved in transfected cells as well in infected cells. As no cleavage products were observed in IP experiments, we decided to detect them, if cleaved, by western blot using antibodies against the N-terminal and C-terminal regions of S. As shown in Fig. 6a, 4 rabbit anti-S antisera were used to detect the S proteins in lysates of Cos-7 transfected cells in western blot experiments. Four major bands were observed when Rb-á-S.1 and Rb-á-S.3 were used (6a, the left two panels) and three when Rb-á- S.2 and Rb-á-S.10 used (the right two panels). All of the major bands are S specific as no similar bands showed in negative controls and also as no bands were observed when Rb-á-S.10 against the C-terminal region of S were used for the shorter, C-terminally truncated S.14 to S.16 (the bottom right panel). The top two bands in each gel are the full-length products of wild type or each deletion mutant. The top bands are 200kD glycosylated S proteins. The second bands from the top are unglycosylated ones as they co-migrated with the corresponding *in vitro* transcription and translation products without canine microsome membranes (CMM). The third and fourth ones in the top two panels might be the N-terminally cleaved products as they are the same in size for the different mutants. The third ones might be the C-terminally cleaved products, because the sizes were decreasing when the smaller constructs were used.

The S proteins in cell lysates and supernatants of virus-infected cells were also detected using Sars S specific antibodies raised in animals and collected from recovered patients in (1) western blot and (2) immunoprecipitation, followed by western blot. The 210kD matured S protein, as well as 125kD and 80kD, proteins were only detected in the supernatant. The 200 kD core-glycosylated S protein, and 3 shorter products 80kD, 95kD, and125kD, were detected in cell lysate. (I will do more, using virus-infected, radiolabeled materials, in next 3 weeks to clarify the size and number of cleavage products.)

ACKNOWLEDGMENTS

We thank Ms Teng Yee Ling, Tham Puay Yoke, Choi Yook-Wah, Hong He and Khu Yee Ling for technique assistance. This work was supported by a grant from the Agency for
Science, Technology and Research (A-Star), Singapore.

REFERENCES

Bosch BJ, van der Zee R, de Haan CA, Rottier PJ. 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J. Virol. 77:8801-11.

Casais R, Dove B, Cavanagh D, Britton P. 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77:9084-9.

Drosten, C., S. Gunther, W. Preiscr, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M, Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M.Sturmer, S. Vieth, H.D. Klenk, A. D. Osterhaus, H. Schmit, H. W. Doerr. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl J. Med. 348:1967-76

Gallagher TM, Buchmeier MJ 2001. Coronavirus spike proteins in viral entry and pathogenesis *Virology* 2001 Jan 20;279(2):371-4

Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A.E. Ling, C.D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, Y. J. Yang, N. Cox, J. M. Hughes, J.W. LeDuc, W. J. Bellini, L. J. Anderson; SARS working group. 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl J. Med. 348:1953-66

Ontiveros E, Kim TS, Gallagher TM, Perlman S. 2003. Enhanced virulence mediated by the murine coronavirus, mouse hepatitis virus strain JHM, is associated with a glycine at residue 310 of the spike glycoprotein. J. Virol. 77:10260-9.

Marra, M.A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M.Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, R. L. Roper. 2003. The genome sequence of the SARS-associated coronavirus. Science. 300: 1399-404.

Rota, P.A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. R. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, W. J.

Bellini. 2003. Characterisation of a novel coronavirus associated with severe acute respiratory syndrome. Science. 300: 1394-9.

Spiga O, Bernini A, Ciutti A, Chiellini S, Menciassi N, Finetti F, Causarono V, Anselmi F, Prischi F, Niccolai N. 2003. Molecular modelling of S1 and S2 subunits of SARS coronavirus spike glycoprotein. Biochem. Biophys. Res. Commun. 310:78-83.

Tsai JC, de Groot L, Pinon JD, Iacono KT, Phillips JJ, Seo SH, Lavi E, Weiss SR. 2003. Amino acid substitutions within the heptad repeat domain 1 of murine coronavirus spike protein restrict viral antigen spread in the central nervous system. Virology 312:369-80.

WE CLAIM:

1. A mature, glycosylated spike protein of a coronavirus.

2. The mature glycosylated spike protein of claim 1 wherein the mature glycosylated spike protein contains a transmembrane domain (TMD).

3. The mature glycosylated spike protein of claim 1 wherein the mature glycosylated spike protein is a 210KDa protein.

4. The mature glycosylated spike protein of claim 2 wherein the coronavirus is a SARS coronavirus.

5. The mature glycosylated spike protein of claim 2 wherein the coronavirus is a SARS coronavirus strain, 2774.

6. A method of producing a mature, glycosylated spike protein of a coronavirus comprising the steps of
   a) transfecting a cell with a nucleic acid encoding a spike protein of a coronavirus or part there of;
   b) expressing the spike protein in the cell; and
   c) isolating the mature, glycosylated spike protein of the coronavirus.

7. The method of claim 6 wherein the mature glycosylated spike protein contains a transmembrane domain (TMD).

8. The method of claim 6 wherein the mature glycosylated spike protein is a 210KDa protein.

9. The method of claim 7 wherein the coronavirus is a SARS coronavirus.

10. The method of claim 7 wherein the coronavirus is a SARS coronavirus strain, 2774.

11. The method of claim 6 wherein the cell is a human lung cell.

12. The method of claim 11 wherein the human lung cell is a lung cell line A549.

13. The method of claim 6 wherein the cell is a cos-7 cell.

14. A method of screening for a mature, glycosylated spike protein of a coronavirus comprising the steps of
   a) isolating a spike protein;
   b) immunoprecipitating the isolated spike proteins with Endo-H; and

c) detecting the remaining spike proteins that are the mature glycosylated spike protein.

15. The method of claim 14 wherein the mature glycosylated spike protein contains a transmembrane domain (TMD).

16. The method of claim 14 wherein the mature glycosylated spike protein is a 210KDa protein.

17. The method of claim 15 wherein the coronavirus is a SARS coronavirus.

18. The method of claim 15 wherein the coronavirus is a SARS coronavirus strain, 2774.

19. An antibody to a mature, glycosylated spike protein of a coronavirus or part their of.

20. The antibody of claim 19 wherein the mature glycosylated spike protein contains a anti-transmembrane domain (TMD).

21. The antibody of claim 19 wherein the mature glycosylated spike protein is a 210KDa protein.

22. The antibody of claim 19 wherein the coronavirus is a SARS coronavirus.

23. The antibody of claim 19 wherein the coronavirus is a SARS coronavirus strain, 2774.

24. A method of detecting a SARS coronaviral infection in a patient comprising the step of applying the antibody of claim 19 to at least part of the cells collected from the patient.

25. A kit for the detection of SARS coronavirus containing the antibody of claim 19.

26. A method to slow the progression of severe acquired respiratory syndrome comprising administering to a patient in need thereof the antibody of claim 19.

27. A vaccine to prevent the onset of severe acquired respiratory syndrome comprising the mature glycosylated spike protein of claim 1.

28. A vaccine to prevent the onset of severe acquired respiratory syndrome comprising the antibody of claim 19.

29. A vaccine to prevent the onset of severe acquired respiratory syndrome comprising the mature glycosylated spike protein of claim 2.

30. A vaccine to prevent the onset of severe acquired respiratory syndrome comprising the antibody of claim 20.

Abstract

The spike (S) protein of Sars coronavirus strain, 2774, was expressed in monkey kidney cells Vero E6 and Cos-7, and in human kidney 293T, lung cells A549 and MRC-5 in a vaccinia-T7 expression system. The S protein was detected by immunoprecipitation (IP), western blot (WB), immunofluorescence (IF), when poly- and mono-clonal antibodies against S, raised in rabbits, horse and mice, were used. These antibodies recognize different regions, covering the whole ectodomain of S. We found that, in a pulse-chase experiment, a 200kD, core-glycosylated form of S was processed into a 210kD, fully glycosylated, endo-H resistant form. Furthermore, when a set of C-terminally-truncated and internal-deletion constructs were expressed, it was found that the S polypeptides without transmembrane domain (TMD) were endo-H sensitive, not transported to the trans-Golgi and not expressed on the cell surface, in contrast to those with TMD. We also found that two host proteins (62kD and 64kD, respectively), in every cell lines tested, were co-immunoprecipitated with S by antibodies raised in animals and antibodies from recovered patients. Amino acid residues 31 to 200 of S were identified to be the domain that interacted with these two proteins, as the deletion of this region completely abolished the specific binding. These two proteins are glycoproteins as they are sensitive to endo-H treatment and they interact with the S protein of Sars coronavirus but not with that of IBV, a member of the group 3 coronaviruses of birds. Interestingly, the S proteins were cleaved into at least two pieces in transfected cell detected in WB experiments, although no conventional cleavage sites were predicted by sequence comparison. In virus-infected cells, similar cleavage products were also observed. The S protein could be detected in the supernatant of infected cells, roughly half of which were cleaved. We found that the most efficient expression and maturation occurred in transfected Cos-7 and A549 cells in parallel transfection experiments. The processing is more complete in A549 cells than in Cos-7 cells, as the majority of the S proteins are the matured, fully-glycosylated 210kD form, which co-migrates with the native form of the S protein in the supernatant of virus infected cells.

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

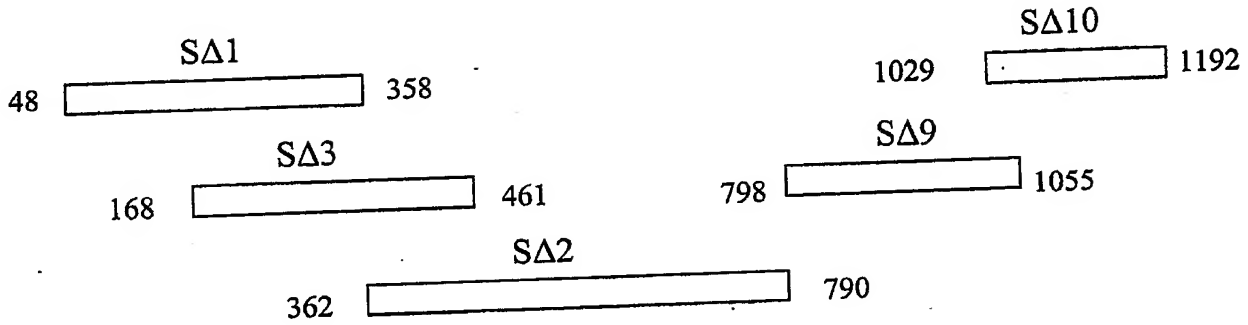
- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Fig. 1

a.


SΔ1
48
358
SΔ10
1029
1192
SΔ3
168
461
SΔ9
798
1055
SΔ2
362
790

b.

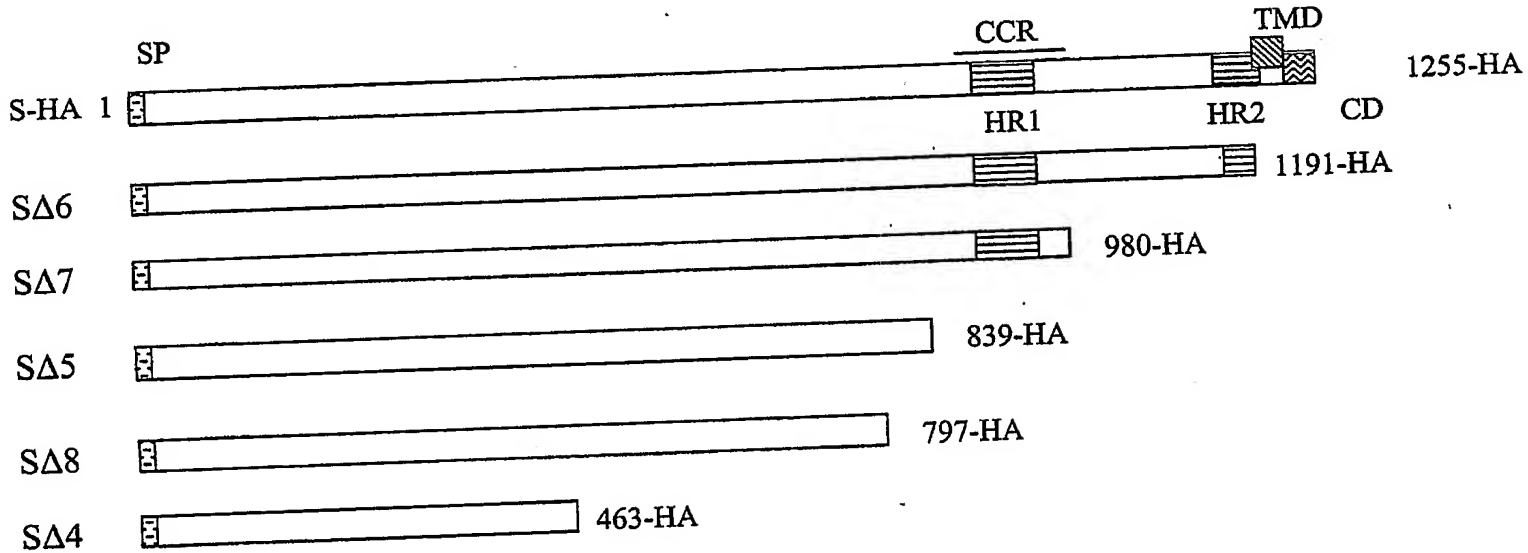

SP
CCR
TMD
S-HA 1
1255-HA
HR1
HR2
CD
SΔ6
1191-HA
SΔ7
980-HA
SΔ5
839-HA
SΔ8
797-HA
SΔ4
463-HA

Fig. 1

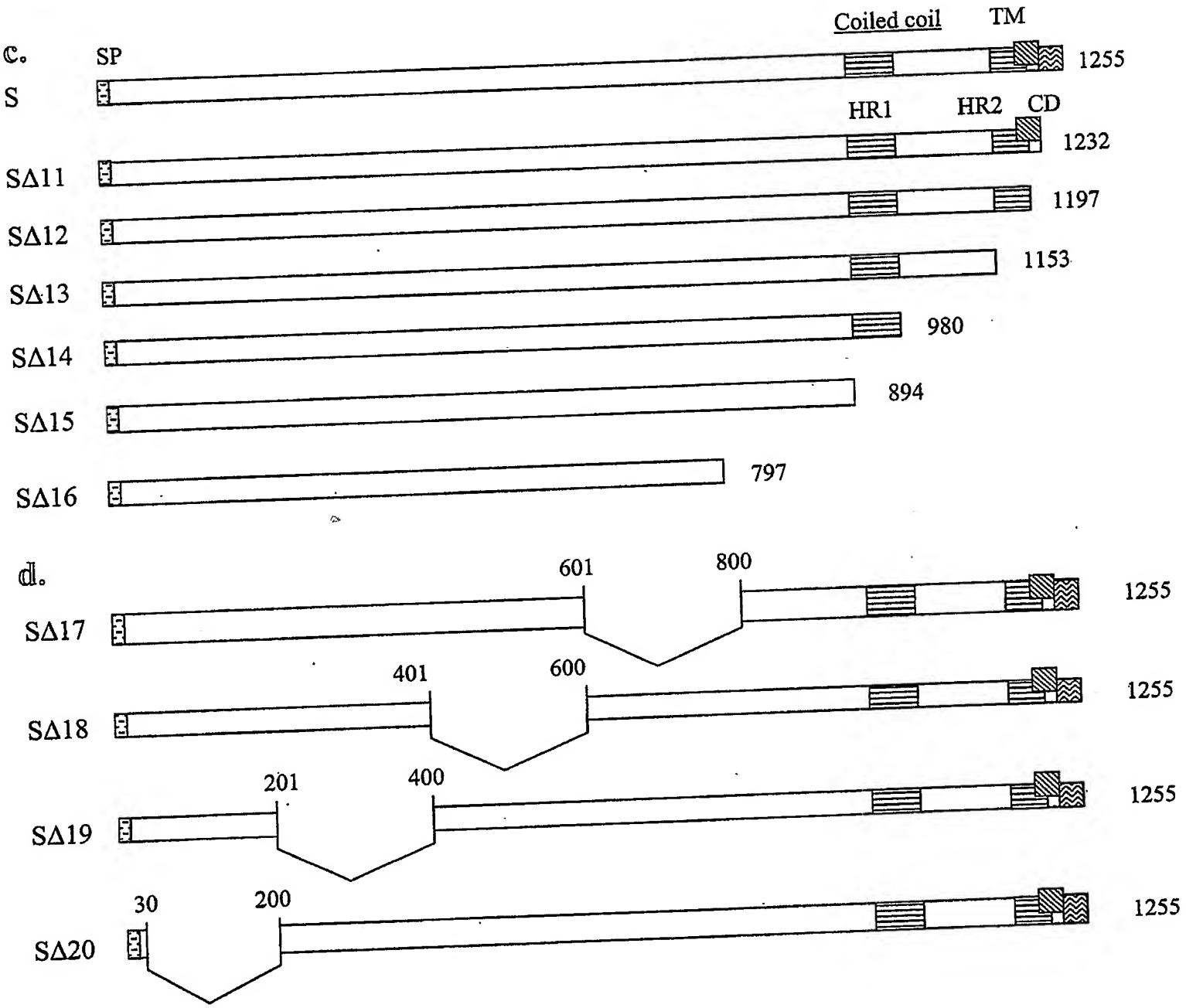

c.
SP
Coiled coil
TM
1255
S
HR1
HR2
CD
1232
SΔ11
1197
SΔ12
1153
SΔ13
980
SΔ14
894
SΔ15
797
SΔ16
d.
601
800
1255
SΔ17
401
600
1255
SΔ18
201
400
1255
SΔ19
30
200
1255
SΔ20

Fig. 2

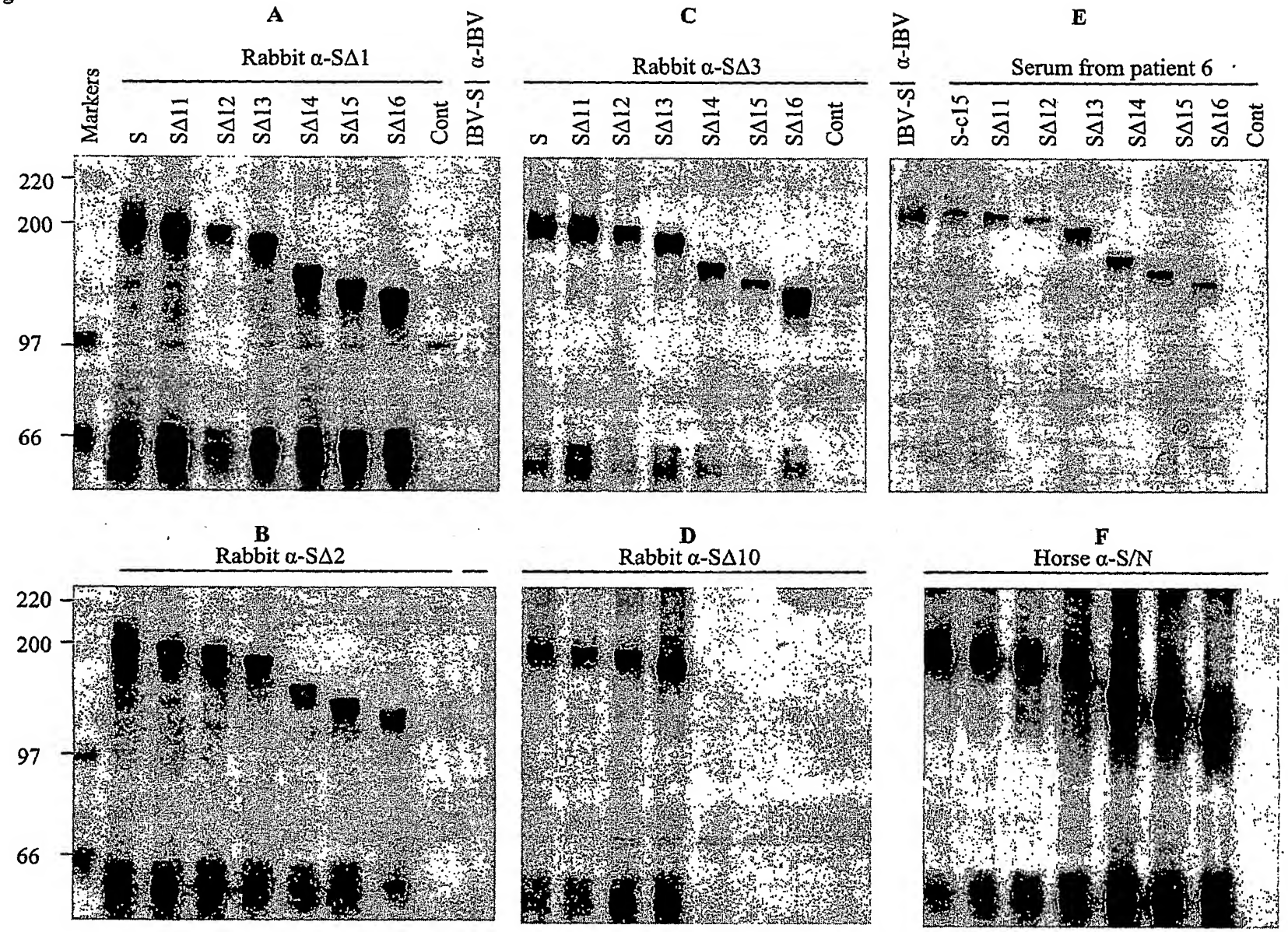

A
Rabbit α-SΔ1
Markers
S
SΔ11
SΔ12
SΔ13
SΔ14
SΔ15
SΔ16
Cont
IBV-S
α-IBV
220
200
97
66
C
Rabbit α-SΔ3
S
SΔ11
SΔ12
SΔ13
SΔ14
SΔ15
SΔ16
Cont
IBV-S
α-IBV
E
Serum from patient 6
S-c15
SΔ11
SΔ12
SΔ13
SΔ14
SΔ15
SΔ16
Cont
B
Rabbit α-SΔ2
220
200
97
66
D
Rabbit α-SΔ10
F
Horse α-S/N

Fig. 3a

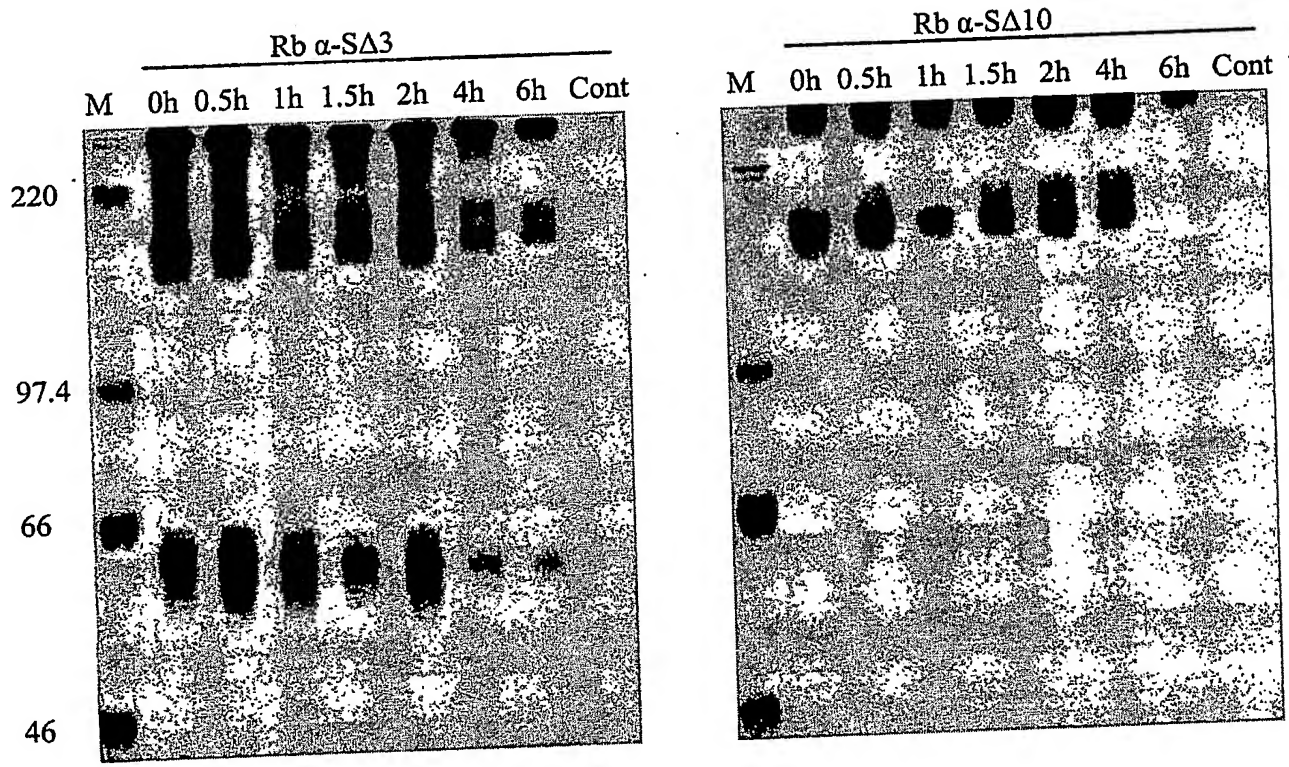

Rb α-SΔ3
M 0h 0.5h 1h 1.5h 2h 4h 6h Cont
220
97.4
66
46
Rb α-SΔ10
M 0h 0.5h 1h 1.5h 2h 4h 6h Cont

Fig. 3b

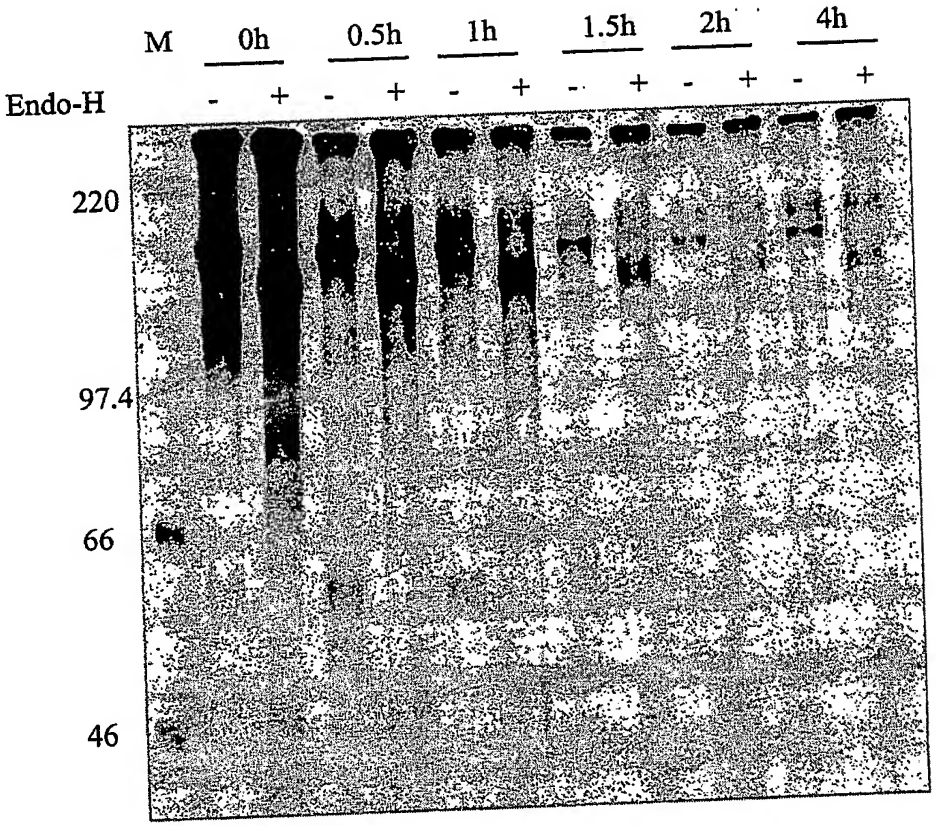


Fig. 4a

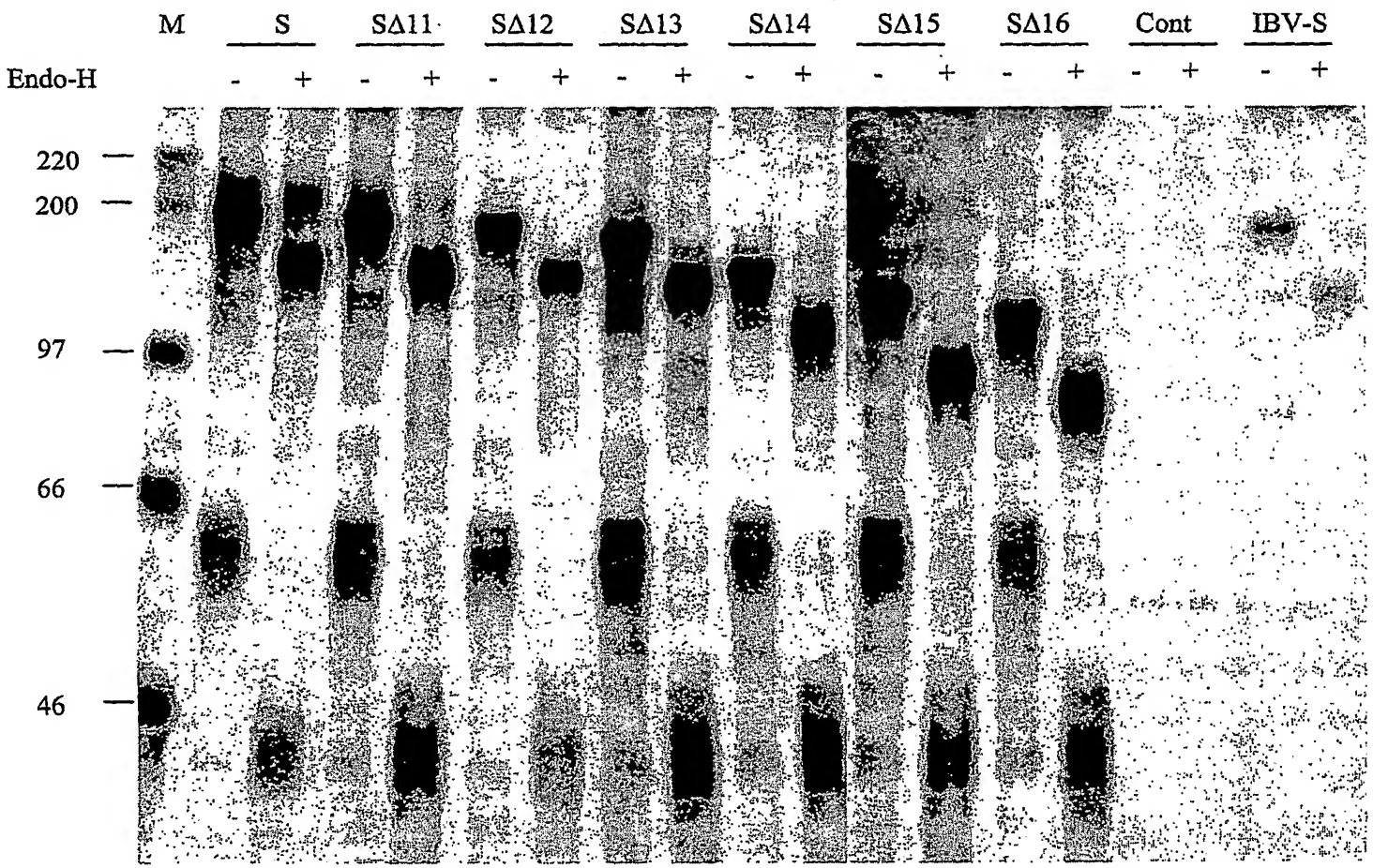

M
S
SΔ11
SΔ12
SΔ13
SΔ14
SΔ15
SΔ16
Cont
IBV-S
Endo-H
- + - + - + - + - + - + - + - + - +
220
200
97
66
46

Fig. 4b

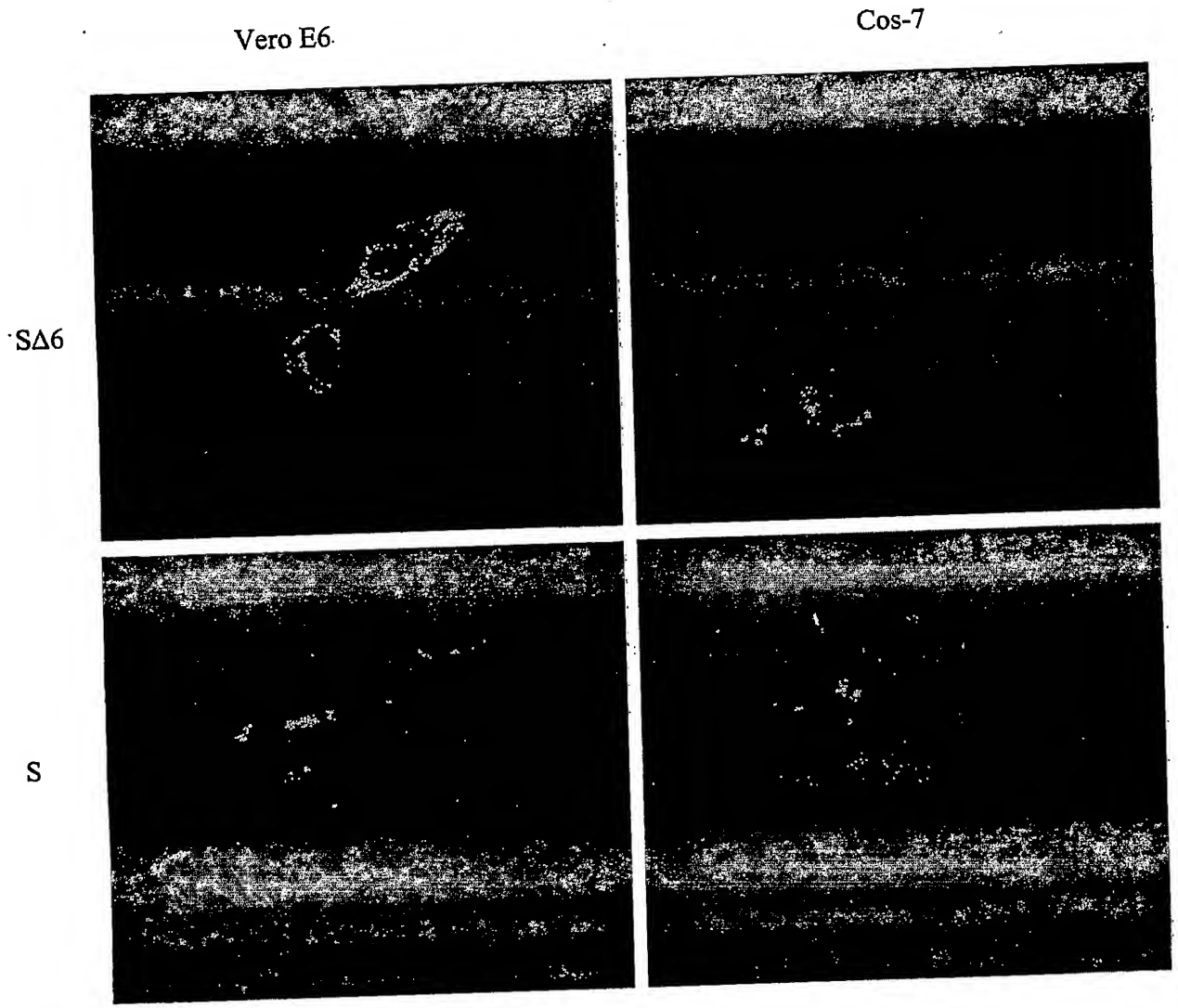


Fig. 5a

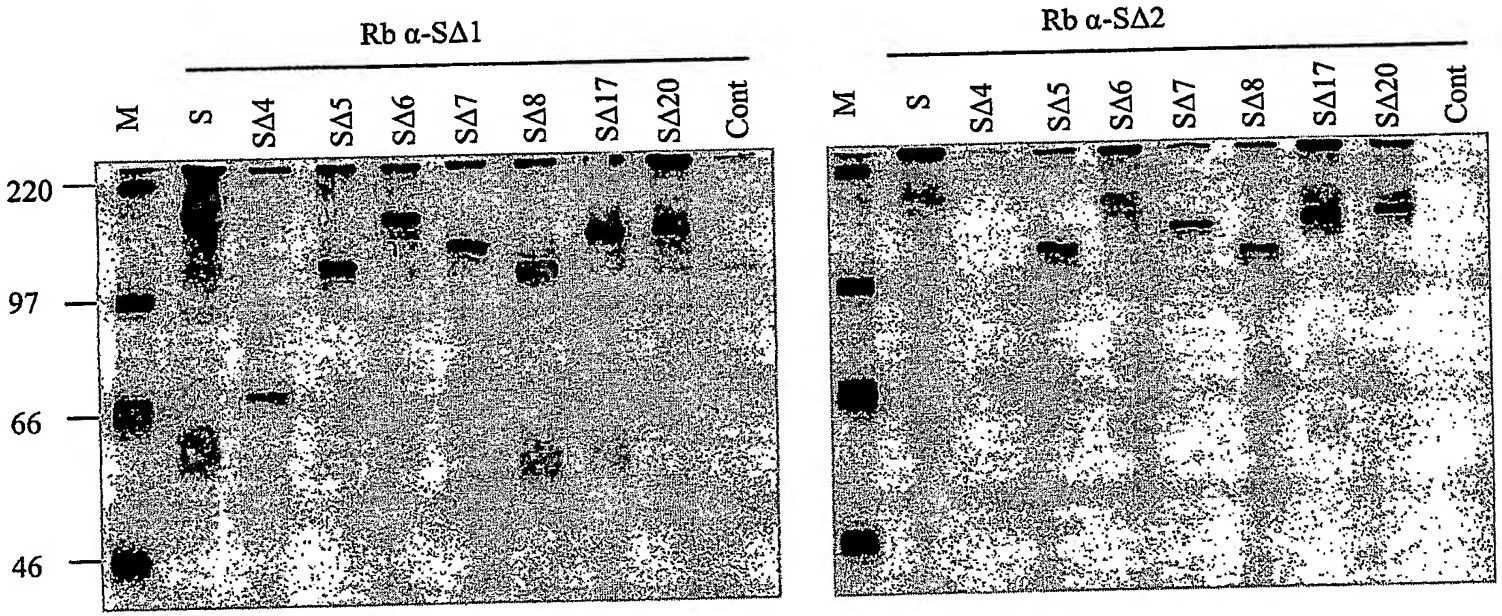


Fig. 5b

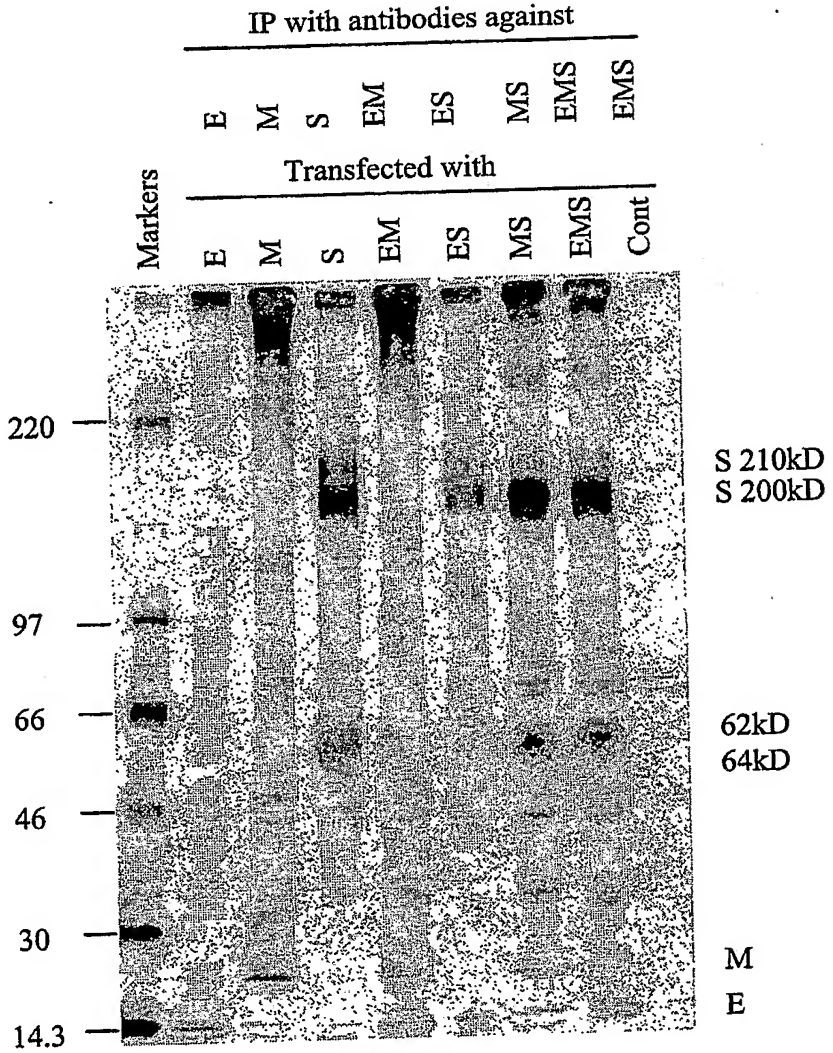

IP with antibodies against
E
M
S
EM
ES
MS
EMS
EMS
Transfected with
Markers
E
M
S
EM
ES
MS
EMS
Cont
220
97
66
46
30
14.3
S 210kD
S 200kD
62kD
64kD
M
E

Fig. 5c

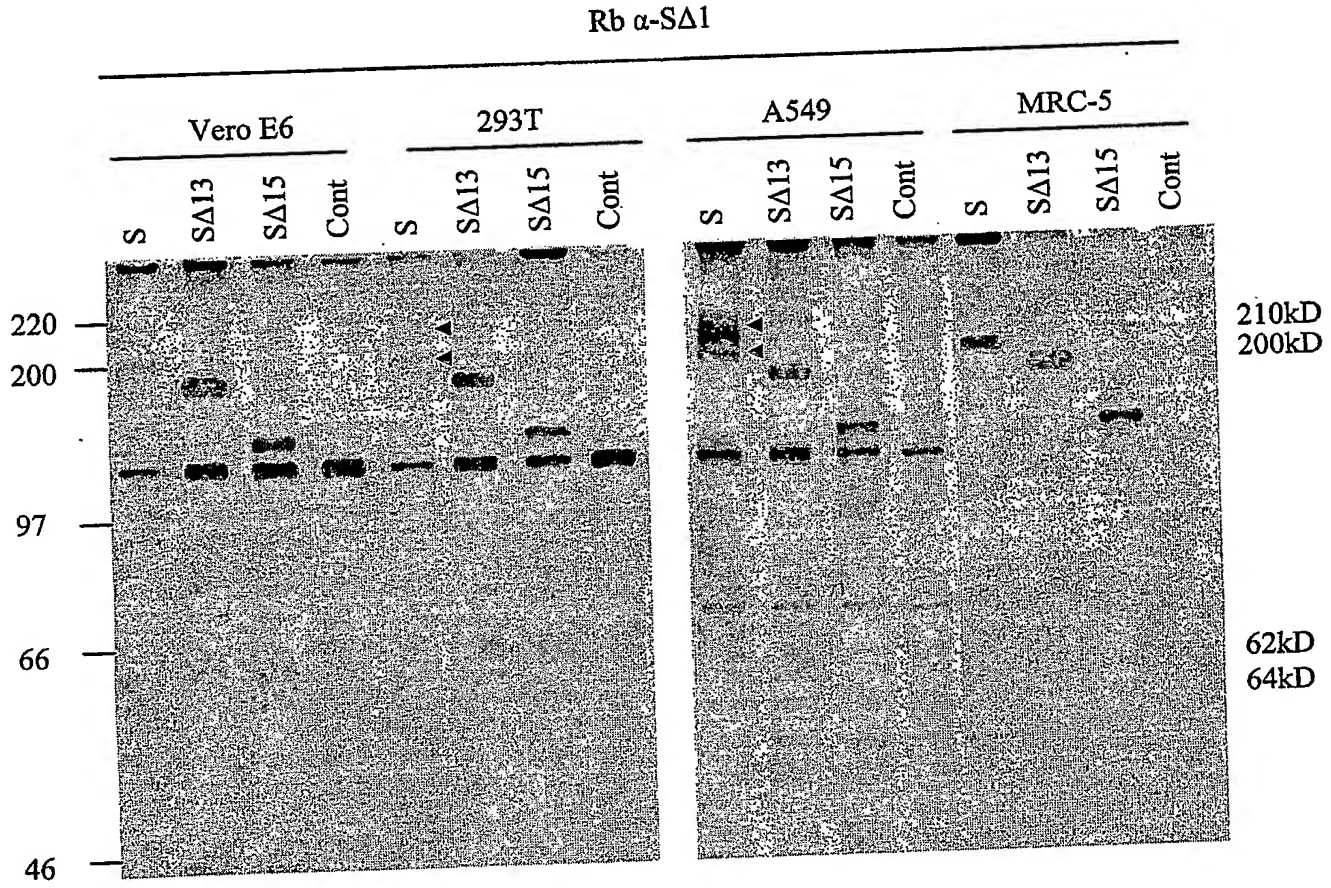

Rb α-SΔ1
Vero E6
293T
A549
MRC-5
S
SΔ13
SΔ15
Cont
220
200
97
66
46
210kD
200kD
62kD
64kD

Fig. 6a

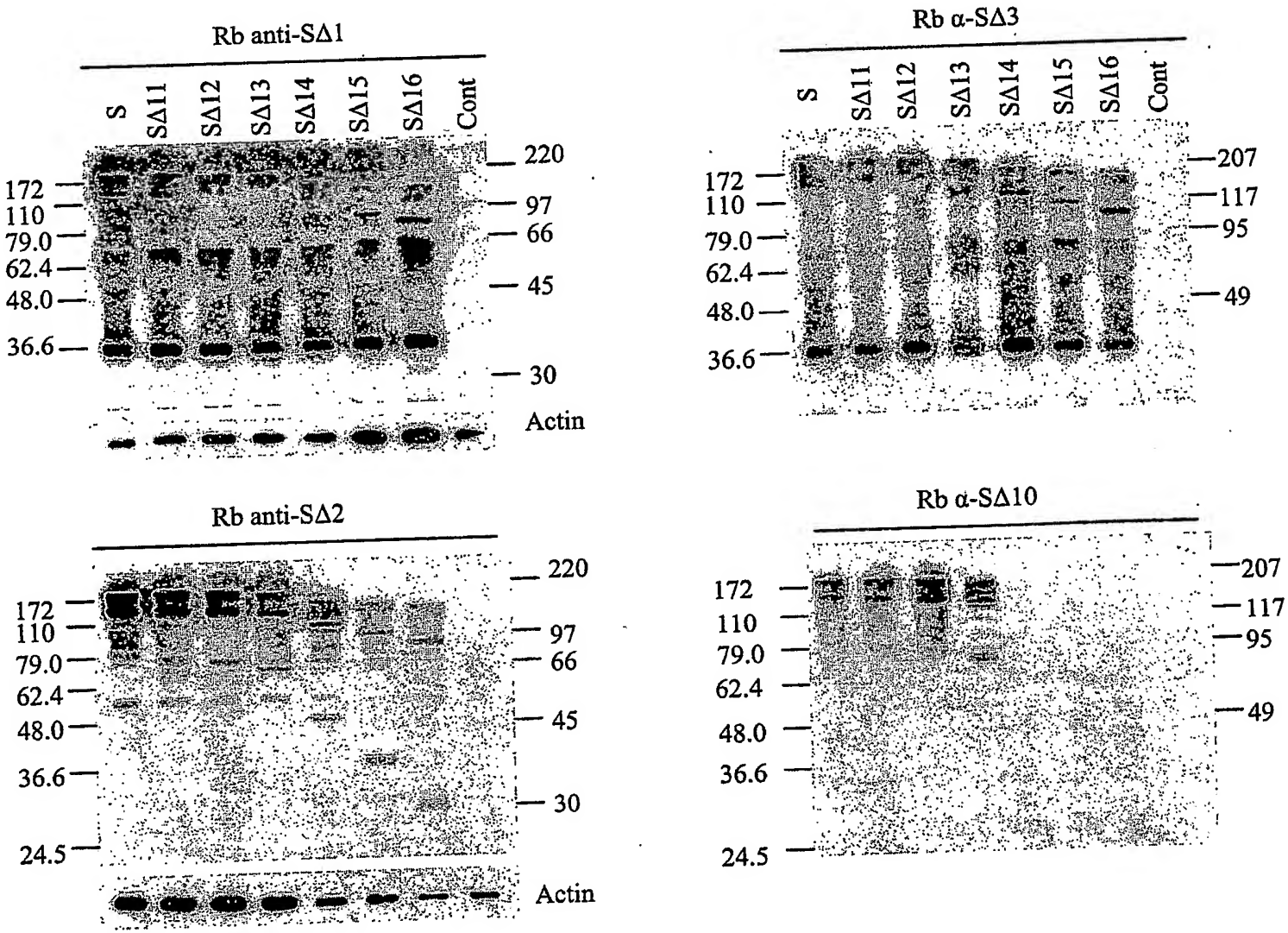

Rb anti-SΔ1
S
SΔ11
SΔ12
SΔ13
SΔ14
SΔ15
SΔ16
Cont
220
172
110
97
79.0
66
62.4
45
48.0
36.6
30
Actin
Rb anti-SΔ2
220
172
110
97
79.0
66
62.4
45
48.0
36.6
30
24.5
Actin
Rb α-SΔ3
S
SΔ11
SΔ12
SΔ13
SΔ14
SΔ15
SΔ16
Cont
207
172
110
117
79.0
95
62.4
49
48.0
36.6
Rb α-SΔ10
207
172
110
117
79.0
95
62.4
49
48.0
36.6
24.5

Fig. 6b

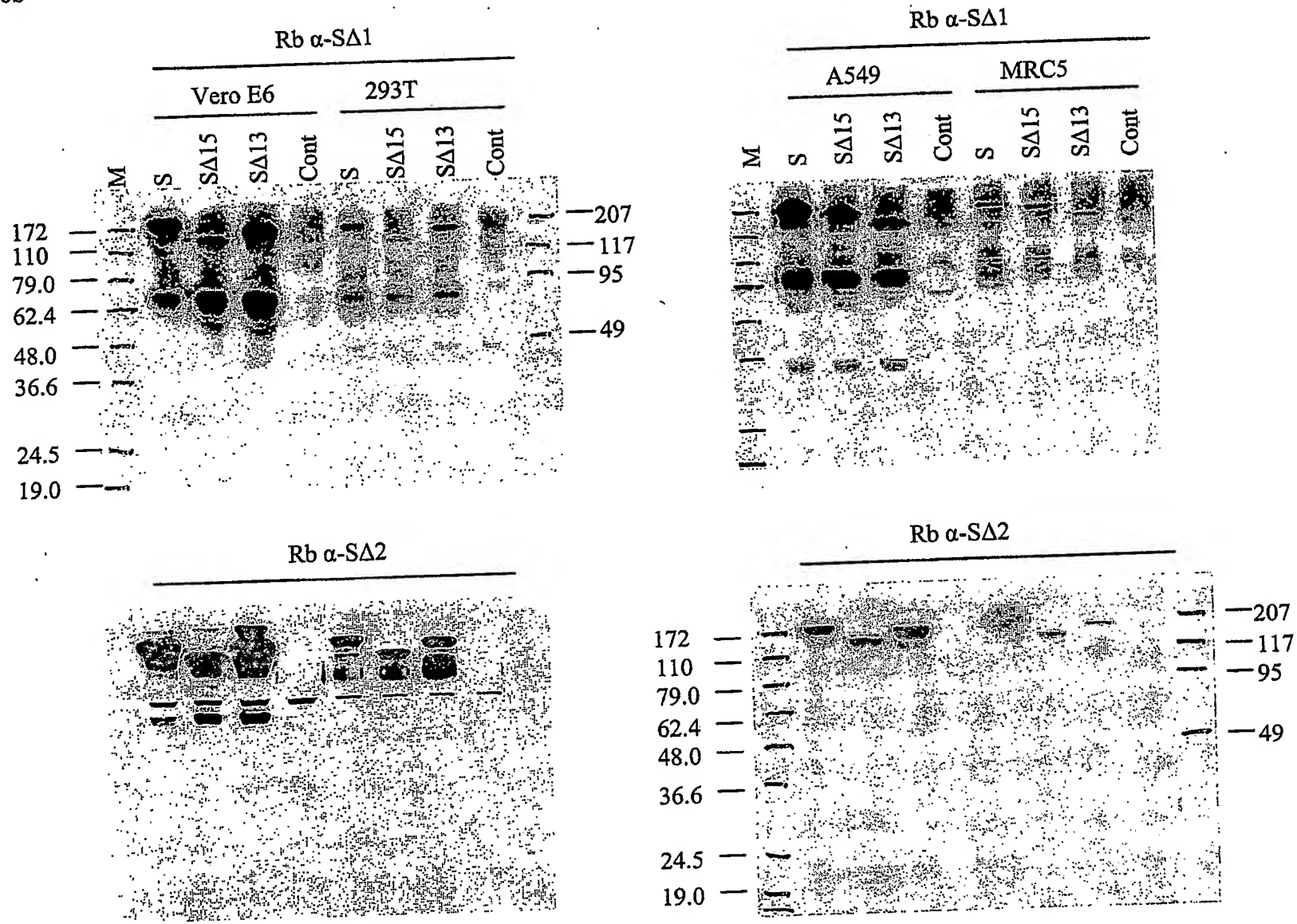
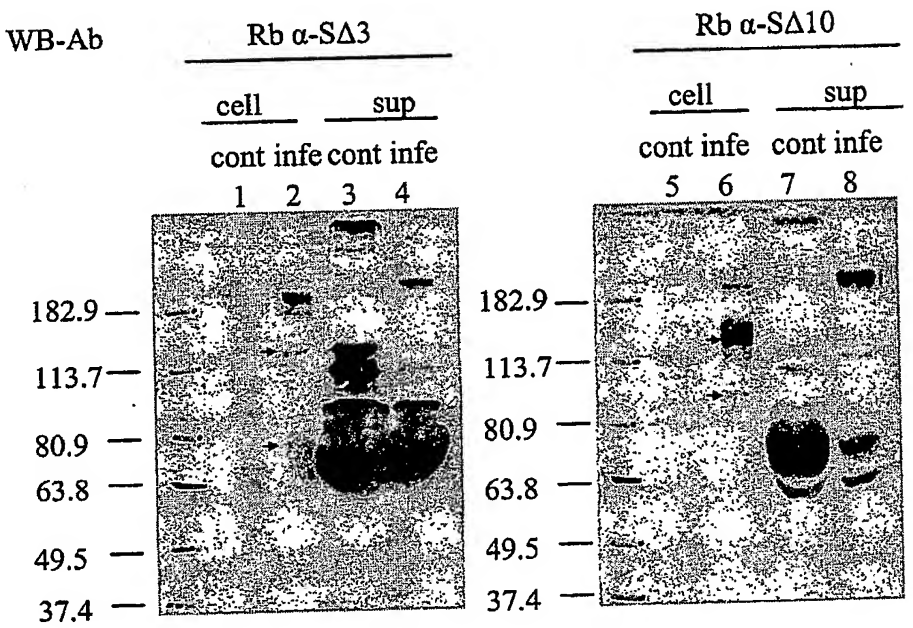
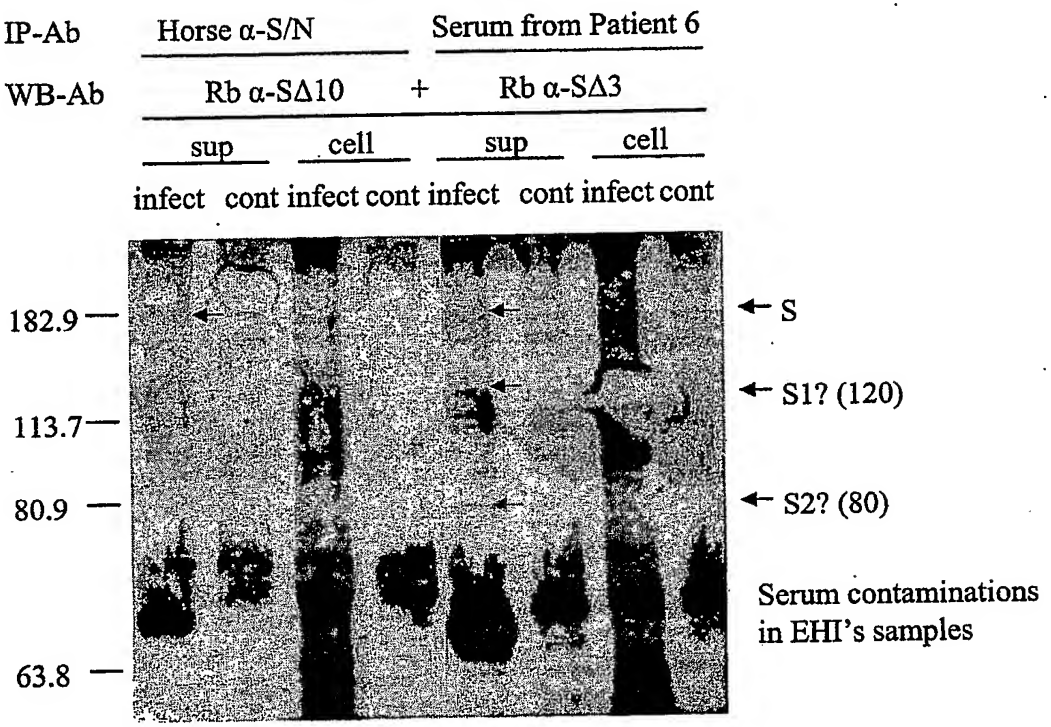

Rb α-SΔ1
Vero E6
293T
M
S
SΔ15
SΔ13
Cont
S
SΔ15
SΔ13
Cont
172
110
79.0
62.4
48.0
36.6
24.5
19.0
207
117
95
49
Rb α-SΔ1
A549
MRC5
M
S
SΔ15
SΔ13
Cont
S
SΔ15
SΔ13
Cont
Rb α-SΔ2
Rb α-SΔ2
172
110
79.0
62.4
48.0
36.6
24.5
19.0
207
117
95
49

Fig. 7a



Matured , 210 kD
Unmatured, 200 kD
Non-glycosylated, 140 kD
Cleaved, 120 (75+40kD in transfected cells) and 80-90 kD ?

Fig. 7b


IP-Ab
Horse α-S/N
Serum from Patient 6
WB-Ab
Rb α-SΔ10
+
Rb α-SΔ3
sup
cell
sup
cell
infect cont infect cont infect cont infect cont
182.9
113.7
80.9
63.8
S
S1? (120)
S2? (80)
Serum contaminations in EHI's samples